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Herpes simplex virus type 1 recombinants without the ori_L sequence replicate DNA with increased fidelity

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Abstract

Herpes simplex virus type 1 (HSV-1) contains three DNA replication origins (*ori*) of two types. The ori_L is located in the center of the unique long sequences, whereas two copies of ori_S, which are structurally different from ori_L, are within the reiterated sequences flanking the unique short sequences. Recombinant viruses were constructed from ts+7, which contains a deletion of ori_L sequences, to have either the β-galactosidase gene or the *supF* amplicon integrated into the thymidine kinase locus. Rescue recombinants also were constructed from the *supF*-containing recombinant to restore the deleted ori_L to the wild type sequences. These recombinants were subjected to mutagenesis assays. Results demonstrated that ts+7 viruses with the deletion in ori_L sequences replicated both target genes with higher fidelity compared to those derived from the parental strain KOS. Possible mechanisms leading to the high fidelity of DNA replication mediated by viruses without intact ori_L sequences are discussed.

Keywords: Herpes simple virus; DNA polymerase; Replication fidelity; Origin of DNA replication; SupF mutagenesis

Introduction

Herpes simplex virus (HSV) contains three copies of the DNA replication origin (*ori*) of two types. One origin is located in the center of the unique long sequences (ori_L), whereas the other two copies, which are structurally different from ori_L, are located within the repeated sequences flanking the unique short sequences (ori_S) (Quinn and McGeoch, 1985; Stow, 1982; Stow and McMonagle, 1983). Although it is not known why HSV maintains three copies of the origin sequences, it has been demonstrated that recombinants lacking either ori_L or both copies of ori_S are able to replicate efficiently in infected cells (Igarashi et al., 1993; Polvino-Bodnar et al., 1987). However, it is unlikely that these sequences are functionally redundant. These two types of origin may have different roles in the viral life cycle, in addition to their roles in the initiation of DNA replication. They are structurally different, despite the extensive sequence similarity. In particular, ori_S comprises an imperfect 45-bp palindrome (Stow and McMonagle, 1983), whereas ori_L is a perfect 144-bp palindrome (Gray and Kaerner, 1984; Quinn and McGeoch, 1985; Weller et al., 1985). Study from Hardwicke and Schaffer (1997) also demonstrates that, in both undifferentiated and nerve growth factor (NGF)-induced differentiated PC12 cells, ori_L-dependent DNA replication is enhanced by dexamethasone (DEX), whereas ori_S function, but not ori_L, is repressed by NGF. These findings suggest the possibility of different roles for ori_L and ori_S in the HSV life cycle, including activities involved in both latent and lytic infections (Hardwicke and Schaffer, 1997). It is also suggested that these sequences may be involved in the temporal regulation of gene expression (Hardwicke and Schaffer, 1997) as ori_L is located upstream of two divergently transcribed early genes, *UL29* and *UL30*, which encodes the major single-stranded DNA binding protein, ICP-8, and the DNA polymerase (Pol), respectively, and ori_S is flanked by immediate-early genes of ICP4 and either ICP22 or ICP47. The presence of two copies of ori_S within the repeat sequences is suggested to be a result of sequence conversion of the repeat sequences (Igarashi et al., 1993).

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The location of ori_L within the viral genome is of particular interest in term of its possible effect on viral DNA replication, since both Pol and ICP-8 play critical roles in viral DNA replication. Mutations within the Pol can affect the fidelity of DNA replication (Hwang and Hwang, 2003; Hwang et al., 1997, 1999, 2002, 2004). ICP8 proteins are found to be associated with DNA repair and recombination proteins in infected cells (Taylor and Knipe, 2004). In this study, we examined whether the absence of ori_L sequences in the HSV-1 virus affected the fidelity of viral DNA replication.

Results and discussions

Construction of Lac Z recombinants

We previously applied the β-galactosidase (*Lac Z*) mutagenesis assay to examine the *Lac Z* mutation frequency replicated in a recombinant tkLTRZ1 (Davar et al., 1994), which has *Lac Z* gene inserted in the thymidine kinase (*tk*) locus, derived from the HSV-1 strain KOS. In order to examine the effect of lacking the ori_L on DNA replication fidelity, the ts+7 mutant virus (Polvino-Bodnar et al., 1987), which contains a deletion of 148-bp within the ori_L sequences, was modified to contain the *Lac Z* gene in its *tk* locus. Such a deletion loses the perfect palindromic structure of the ori_L and results in the failure of initiating the origin-dependent DNA replication in the transient DNA replication assay (Polvino-Bodnar et al., 1987). Two independent recombinants, ts+7/Lac Z-A and -B (Fig. 1A), were isolated from independent transfection experiments and twice plaque-purified to homogeneity. Southern analysis confirmed that both ts+7/Lac Z recombinants contained the *Lac Z* gene inserted into the *tk* locus (Fig. 1B). A probe, consisting of the *Pst*I 840-bp fragment of the *tk* gene (Fig. 1A), hybridized to an 840-bp band, corresponding to the *Pst*I-digested, of wild type KOS DNA (Fig. 1B, left panel, lane 2). A shift from the 840-bp band to a 6.4-kb band was detected in *Pst*I-digested DNA isolated from tkLTRZ1, ts+7/Lac Z-A, and -B DNA (Fig. 1B, left panel, lanes 1, 3, and 4, respectively). The *Mlu*I 780-bp probe, prepared from the *Lac Z* gene (Fig. 1A), failed to detect a hybridization signal from *Pst*I-digested KOS DNA (Fig. 1B, right panel, lane 2), while a 6.4-kb band was detected in *Pst*I-digested DNA from tkLTRZ1, ts+7/Lac Z-A, and -B (Fig. 1B, right panel, lanes 1, 3, and 4, respectively). Both probes hybridized to plasmid DNA at the correct sizes (Fig. 1B, right panel, lane 5 and Fig. 1B, right panel, lane 5). Therefore, both ts+7/Lac Z-A and -B recombinants contained the *Lac Z* gene and were homogeneous.

Lac Z mutagenesis assay

Single plaques were isolated from ts+7/Lac Z-A and -B, amplified in Vero cells, and designated as viral stocks. *Lac Z* mutagenesis was performed to examine the mutation frequency of the *Lac Z* gene as described in Materials and methods. Recombinant stocks of ts+7/Lac Z-A and -B exhibited mutation frequencies of 0.01% and 0.008%, respectively (Table 1), which were lower than the frequencies observed for tkLTRZ1 in infected cells (0.015%) (Hwang et al., 2003). The low *Lac Z* mutation frequency replicated by ts+7 recombinants is surprising. Because of the low mutation frequency, it is necessary to examine a larger number of progeny viruses in order to reveal a more accurate frequency. However, a bias may be resulted from the isolation of sister mutants derived from the same mutants. Therefore, a different approach was used. Three plaques were isolated from each ts+7/Lac Z recombinant virus, amplified, and subjected to mutagenesis assay. It is noted that a viral inoculation with 100–200 pfu should not give rise to inoculation with a pre-existing mutant. Progeny viruses were harvested at 72 h post-infection and their titers were determined. Results demonstrated that ts+7/Lac Z recombinants replicated the *Lac Z* gene with a significantly higher fidelity ($P < 0.05$ and $P < 0.001$ for ts+7/Lac Z-A and -B, respecitively) than tkLTRZ1, with mutation frequencies ranging from 2- to 10-fold less (Table 1). Although ts+7/Lac Z-B recombinant replicated with a lower mutation frequency relative to that of ts+7/Lac Z-A virus, the difference is not statistically significant ($P = 0.1846$).

Analyses of UL29 and UL30 genes

Both viral DNA polymerase (UL30) and ICP-8 proteins (UL29) are essential for viral DNA replication and located divergently to the ori_L. Additionally, experimental data have demonstrated that mutations in the catalytic subunit of HSV-1 DNA polymerase, either in the polymerase or exonuclease domains, have been shown to have a direct effect on replication fidelity (Hwang and Hwang, 2003; Hwang et al., 1997, 1999, 2002, 2004). It is possible that ts+7-derived recombinants may acquire mutations in both genes, resulting in higher fidelity. To rule out this possibility, we sequenced both the *UL29* and *UL30* genes using either the cloned DNA fragments (the *UL29* gene) or the PCR-amplified products (the *UL30* gene). The plasmids pGEM-ts+7/Lac Z-UL29-A and -B, which contain the entire *UL29* open reading frame, ori_L, and the 5′ portion of *UL30*, were constructed from ts+7/Lac Z-A and -B, respectively, and sequenced as described in Materials and methods. PCR-amplified *UL30* sequences also were sequenced as previously described (Hwang et al., 2002, 2004). Sequencing results did not discover any changes in the coding sequences of both genes, compared to those of KOS (Gao et al., 1988; Gibbs et al., 1985). However, an insertion of 2 Cs in the polynucleotide runs of 11 Cs, resulting in 13 Cs, located upstream of the *UL29* coding sequence was found (data not shown). It also is noted that strain 17 contains the runs of only 9 Cs at this region (McGeoch et al., 1988), suggesting that the difference may be a polymorphism. Sequencing of both UL29 and UL30 genes also demonstrated that the ts+7 mutants and the derivatives are derived from the strain KOS. Therefore, the altered mutation frequency observed in ts+7 recombinants does not appear to be the result of the mutation of *UL29* and *UL30* genes. However, we do not rule out the possibility that these recombinants may acquire mutation in other genes, which are essential for viral DNA replication and may play a role affecting the replication fidelity (see below). Therefore, experiments with another reporter were carried out to further test replication fidelity.

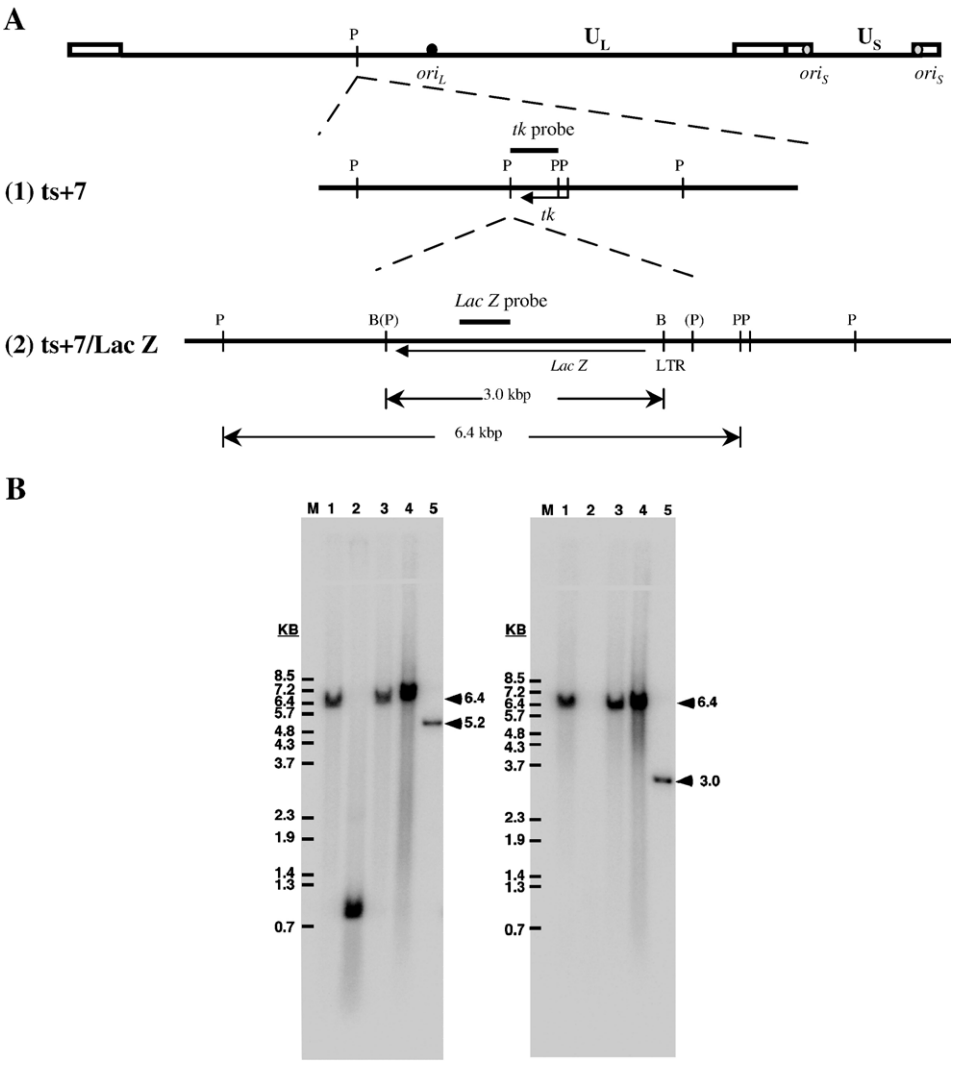

Fig. 1. Maps and Southern blot analysis of ts+7/Lac Z recombinant. (A) Map of ts+7 and ts+7/Lac Z recombinants. The top line denotes the genome of HSV-1 with the relative locations of three origins of DNA replication (ori_L and ori_S). Line 1, designated as ts+7, shows an enlarged region flanking the HSV-1 *tk* locus with the relative *Pst*I restriction sites. The relative position of the *tk* probe used for Southern analysis is also shown. Line 2, designated as ts+7/Lac Z, shows the relative position of the inserted *Lac Z* gene, which is under the control of the MoMLV LTR promoter. The relative *Bam*HI restriction sites of the *Lac Z* DNA are also shown. The insertion of the *Lac Z* construct that results in the destruction of the *Pst*I site in the *tk* locus is indicated by parentheses. The relative locations of the *tk* and *Lac Z* probes are shown. The corresponding size of the *Bam*HI and *Pst*I fragments, present in ts+7/lac Z recombinants, are shown on the bottom lines. (B) Southern blot analyses. Southern blot analysis using the *tk Pst*I 849-bp probe is shown on the left panel. Lanes 1 to 4 depicts *Pst*I-digested genomic DNA isolated from Vero cells infected with tkLTRZ1, KOS, ts+7/Lac Z-A, and -B, respectively. Lane 5: *Bam*HI-digested ptkLTRZ1 plasmid DNA (control). The sizes of the molecular weight markers are shown on the left. Southern analysis using the *Lac Z* 780-bp *Mlu*I probe is shown in the right panel. Lanes 1 to 4 depict *Pst*I-digested DNA isolated from Vero cells infected with tkLTRZ1, KOS, ts+7/Lac Z-A, and -B, respectively. Lane 5: *Bam*HI-digested ptkLTRZ1 plasmids DNA (control). The sizes of the molecular markers are shown on the left.

SupF mutagenesis assay

The *SupF* reporter gene, which had been applied for the studies of the effects of *pol* mutations in the replication fidelity, was chosen for the next sets of experiments. Two independent *SupF*-containing recombinants, ts+7/F-A and -C, were constructed, isolated, and purified under 10 μM gancyclovir (GCV) selection as described previously (Hwang and Hwang, 2003). Southern blot analysis confirmed the insertion of the *SupF* amplicon in the *tk* locus and demonstrated the homogeneity of these recombinants (data not shown). Single plaques of each recombinant virus were isolated by plaque assay, amplified in Vero cells, and designated as viral stocks. To determine the *SupF* mutants present in these viral stocks, *SupF* mutagenesis assay was performed and the relative mutation frequency was determined. The mutation frequencies were determined to be less than 0.001% and 0.0012% for the stock of ts+7/F-A and -C recombinants, respectively (Table 2). Approximately 100–200 pfu of each virus were inoculated into 1×10^5 Vero cells, incubated for 72 h, and the mutation frequencies were determined. Experiment 1, shown in Table 2, illustrates that both ts+7/F recombinants replicated with an approximate 5-fold lower mutation frequency compared to KOS/F-B virus ($P < 0.001$), which is derived from strain KOS and contains an identical *SupF* amplicon in the *tk* locus (Hwang et al., 2002).

Table 1
Lac Z mutation frequency of ts+7/Lac Z recombinants

Virus	Clear plaques/ Total plaques	Mutation frequency, % (*P* value)[a]
Stock		
tkLTRZ1[b]	0/2142	<0.047
ts+7/Lac Z-A	1/9509	0.010
ts+7/Lac Z-B	1/11,985	0.0083
Experiment		
tkLTRZ1	5/27,180	0.018
ts+7/Lac Z-A1[c]	2/25,876	0.0077
ts+7/Lac Z-A2	2/50,607	0.0040
ts+7/Lac Z-A3	1/19,850	0.0050
Sum	5/96,333	0.0052 (<0.05)
ts+7/Lac Z-B1	1/35,950	0.0028
ts+7/Lac Z-B2	1/53,994	0.0019
ts+7/Lac Z-B3	1/57,210	0.0017
Sum	3/147,154	0.0020 (<0.001)

[a] Chi-square test was used to determine the statistical significance.
[b] Data from reference Hwang et al. (2003) and assayed on Vero cells.
[c] Three different plaques were isolated, amplified on Vero cells, and subjected to mutagenesis study.

To rule out the possibility that mutation in UL29 and UL30 could contribute to the altered replication fidelity, both *UL29* and *UL30* genes were cloned and PCR-amplified, respectively, and sequenced as described above. Results did not reveal any changes in the coding sequences of both genes (data not shown) with the exception of the inserted 2 Cs in the non-coding sequences upstream of the *UL29* open reading frame, identical to that found in ts+7/Lac Z recombinants. It, however, remains a possibility that these recombinants may acquire mutation in other replicative proteins, which affect the replication fidelity. For example, mutation in *UL42*, the processivity factor of the catalytic subunit of Pol, could mediate an altered DNA replication fidelity (Jiang and Hwang, unpublished data). Studies from other system also demonstrate that mutations in gene encoding proliferating cell nuclear antigen (PCNA), the processivity factor of Pol δ, can induce an increase of mutation frequency in vivo (Chen et al., 1999).

Construction of rescue recombinants

To rule out the possibility that the higher fidelity of DNA replication mediated by ts+7 recombinants was due to a second mutation in other genes of recombinants, experiments were designed to rescue the partially deleted ori_L sequences. Since there is no direct approach to select and isolate rescue recombinants, we took a two-step approach to revert the partially deleted ori_L to its wild type counterpart using two newly constructed plasmids described below. This was done first by the construction of a recombinant to contain the deletion of ori_L and partial *pol* sequences, and an insertion of DsRed2 gene expressing Red fluorescent protein. The altered sequences allow a rapid screening of recombinants expressing Red fluorescent marker, which can only propagate in a Pol cell line because of the deletion in *pol* gene. The second recombinant will be rescued to contain the wild type sequences of both ori_L and *pol* sequences and can be isolated as that lacking the expression of Red fluorescent marker and restoring the ability to grow on Vero cells.

A recombinant, ts+7/F-Red2 (Fig. 2, line 4), with a deletion of the N-terminal part of *UL30* and an insertion of a fluorescent *DsRed2* marker, was constructed by transfection of pUL29/30-Red2 into PolA5 cells, followed by superinfection with ts+7/F-C recombinant virus. The resulting recombinant, ts+7/F-Red2, which could only grow on PolA5 cells and express red fluorescent protein, was isolated using fluorescent microscopy. Recombinant ts+7/F-Red2 was purified to homogeneity. PCR reaction using oligonucleotide primers ICP8-2 and Red-545 demonstrated that the *DsRed2* gene was present in recombinant ts+7/F-Red2 (Fig. 3B, lane 3), but not in wild type KOS/F-B or ts+7/F-C (Fig. 3B, lanes 1 and 2, respectively). An internal control, which amplified a 564-bp fragment of the *UL42* gene, was included in all reactions to rule out PCR failure in the absence of a *DsRed2* PCR product. Primers UL42-2 and UL42-8 were used in this experiment. This PCR experiment also demonstrated the homogeneity of rescue-A and -B recombinants by the lack of *DsRed2* PCR products (see below; Fig. 3B, lanes 4 and 5).

Two independent rescue mutants, rescue-A and -B, were then constructed by transfection of the plasmid pKS-UL29/30 into PolA5 cells, followed by superinfection with ts+7/F-Red2 recombinant virus. These constructs were identified by the loss of DsRed2 expression and the ability to grow on

Table 2
SupF mutation frequencies replicated by ts+7/F recombinants

Virus	White colonies/Total colonies (mutation frequency, %)	(*P* value[a])
Stock		
KOS/F-B[b]	89/348,610 (0.026)	
ts+7/F-A	0/78,855 (<0.001)	
ts+7/F-C	1/82,539 (0.0012)	
Rescue-A	3/13,791 (0.021)	
Rescue-B	3/62,374 (0.0048)	
Exp. 1		
KOS/F-B	11/87,548 (0.013)	
ts+7/F-A	6/282,083 (0.0021)	(<0.001)
ts+7/F-C	6/235,947 (0.0025)	(<0.001)
Exp. 2		
KOS/F-B	8/82,774 (0.0097)	
ts+7/F-A	8/303,180 (0.0026)	(<0.01)
ts+7/F-C	2/99,533 (0.0020)	(<0.05)
Rescue-A	34/277,005 (0.0123)	(<0.05)[c]
Rescue-B	11/106,091 (0.0104)	(<0.01)[c]
Exp. 3		
KOS/F-B	5/39,018 (0.013)	
ts+7/F-A	4/144,107 (0.0028)	(<0.05)[d]
ts+7/F-11C	2/70,061 (0.0029)	(<0.05)[d]

[a] Chi-square test was used to determine the *P* values for mutation frequency, compared to that of KOS/F.
[b] Data from reference Hwang et al. (2002).
[c] The *P* value for mutation frequency was compared to that of ts+7/F-C.
[d] The *P* value for mutation frequency was compared to that of KOS/F-B.

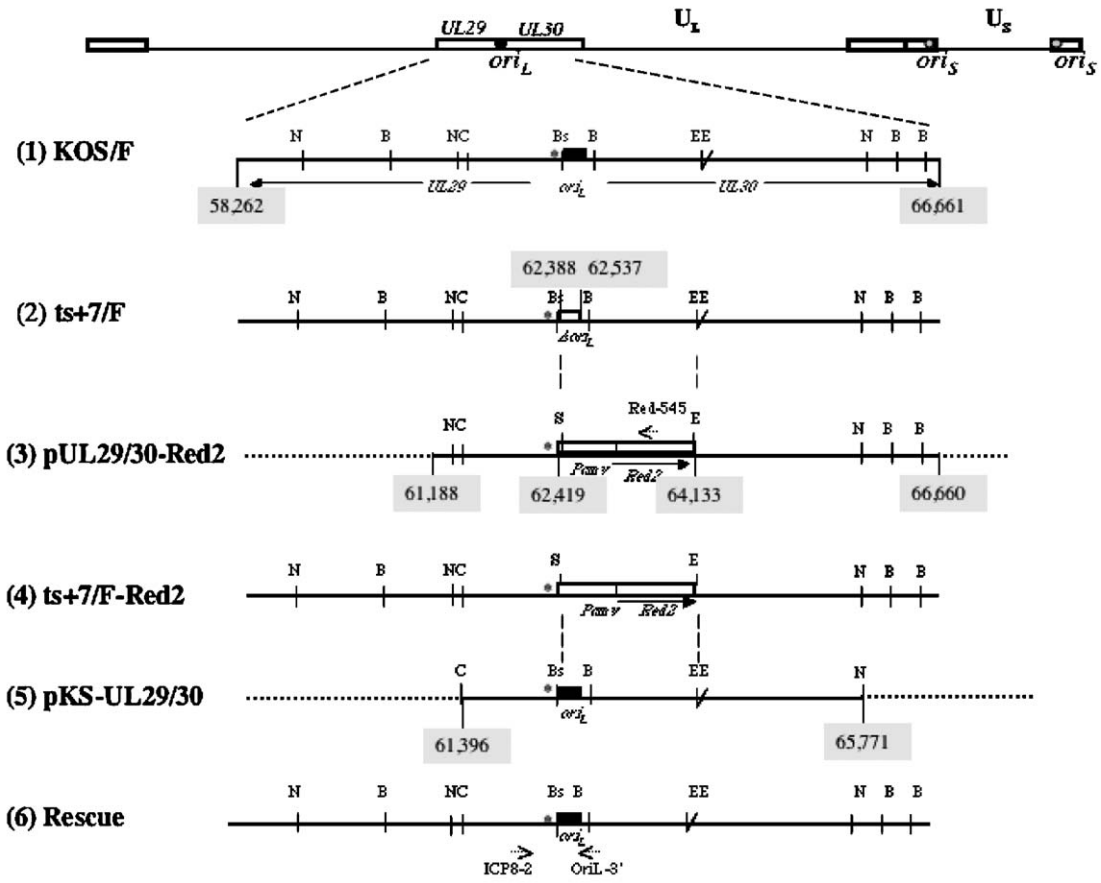

Fig. 2. Map of *supF* recombinants and plasmids. The top line denotes the genome of HSV-1 with the relative locations of the three origins of DNA replication (ori_L and ori_S), *UL29*, and *UL30* genes. Line 1 shows the relative restriction map spanning the *UL29* gene, wild type ori_L (closed box), the *UL30* gene of KOS/F recombinants. Line 2 shows the relative location of the deleted ori_L (opened box) in ts+7/F recombinants. Line 3 shows the relative map of the plasmid pUL29/30-Red2, which contains the N-terminal portion of *UL29*, partial ori_L region, *Pcmv-DsRed2*, and the C-terminal portion of *UL30*. Line 4 shows the relative map of recombinant virus ts+7/F-Red2, which contains the *Pcmv-DsRed2* cassette inserted between *UL29* and *UL30*. Line 5 shows the relative map of the plasmid pKS-UL29/30, which contains the N-terminal portion of *UL29*, the entire ori_L, and the N-terminal portion of *UL30*. Line 6 shows the relative map of rescue-A and -B recombinants, which contains the rescued ori_L sequence (closed box). Numbers depict the corresponding nucleotide positions in the HSV-1 genome, according to McGeoch et al. (1988). The open reading frames of *UL29*, *UL30*, and *DsRed2* are shown as arrows with solid lines. The relative locations of primers Red-545, ICP8-2, and oriL-3′ are shown as arrows with dotted lines. The symbol of * depicted the relative location of the runs of Cs. Restriction enzymes depicted are: B, *Bam*HI; Bs, *Bsr*GI; C, *Cla*I; E, *Eco*RI; N, *Not*I; P, *Pst*I; S, *Sph*I.

Vero cells, since recombination would result in acquiring the wild type sequences of ori_L and *pol*. As seen in Fig. 3B (lanes 4 and 5), both rescue recombinants appeared to lose the Pcmv-DsRed2 sequence as shown by a lack of *Red2* amplification. To further confirm that these rescue recombinants contain the intact ori_L sequences, oligonucleotide primers ICP8-2 and oriL-3′, corresponding to sequences located upstream and downstream of the ori_L sequence (Fig. 1B, line 6), were used to amplify the corresponding sequences from DNA isolated from virus-infected cells. A 662-bp fragment was amplified from rescue-A and -B (Fig. 3C, lanes 4 and 5, respectively), consistent with the results from KOS/F-B (Fig. 3C, lane 1). This result is in sharp contrast to the 514-bp DNA fragment amplified from ts+7/F-A and -C viral DNA (Fig. 3C, compare lanes 1, 4, and 5 with 2 and 3). Two *Psi*I restriction sites are shown to exist in the 148-bp region that is the deleted component of the palindromic sequence of ori_L in the ts+7 genome (Fig. 3A). The presence of these restriction sites offers the advantage of a quick diagnosis for the presence of ori_L. The PCR-amplified 662-bp fragment was digested with *Psi*I into three fragments of 409, 157, and 96 bp (Fig. 3C, lanes 6, 9, 10), suggesting the presence of full-length ori_L, whereas the products of ts+7/F recombinants were not cleaved by *Psi*I (lanes 7 and 8). Furthermore, the multiple bands observed in undigested DNA disappeared after *Psi*I digestion, suggesting that these multiple bands are due to the secondary structures formed by palindrome sequences. The presence of wild type ori_L sequences in rescue-A and -B recombinants was further confirmed by direct sequencing of the PCR products that were amplified from these viral DNAs (data not shown). Both *UL29* and *UL30* genes were cloned and PCR-amplified, respectively, and sequenced as described above. Results did not reveal any changes in both genes, including the runs of 11 Cs of the strain KOS sequence in the non-coding sequences (data not shown).

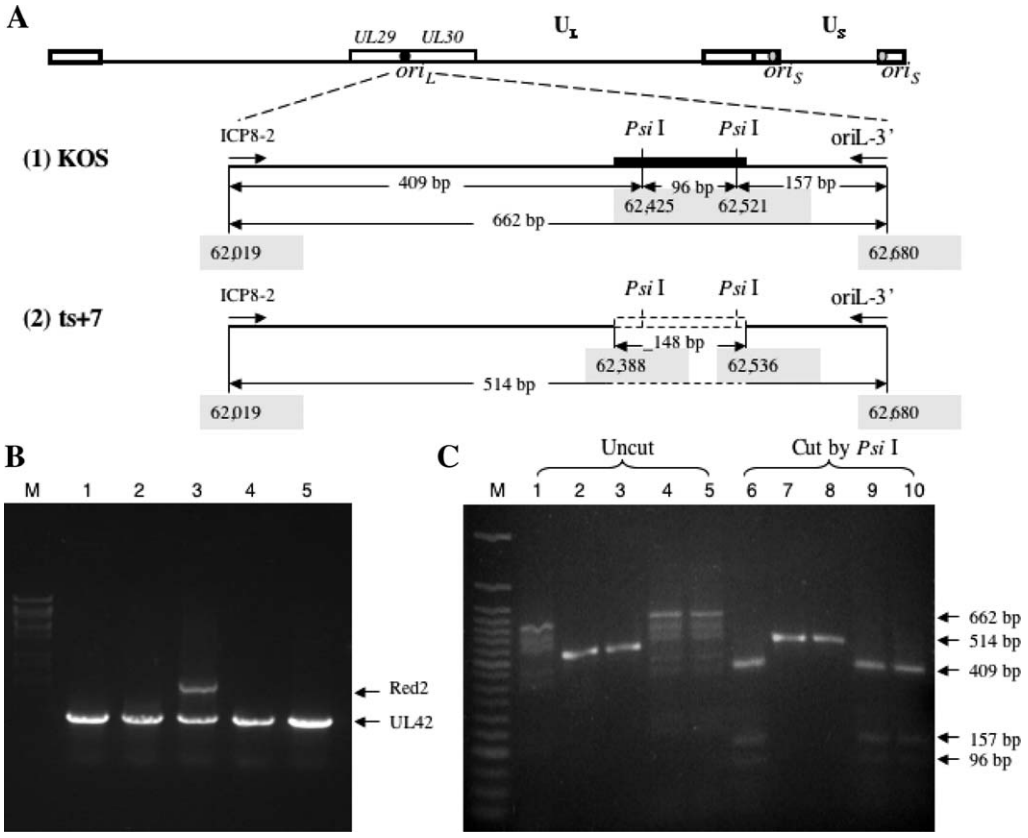

Fig. 3. Analysis of rescued recombinants. (A) Map of sequences spanning the ori_L region. The line, designated as KOS, shows the map of 662-bp DNA fragment amplified from viral DNA containing the intact ori_L sequence using primers ICP8-2 and OriL-3′. The line, designated as ts+7, shows the map of a 514-bp fragment from ts+7 recombinants using the same primers. The relative size of the DNA fragment after *Psi*I digestion is shown. The closed box (in KOS) depicts the 148-bp fragment within the ori_L sequence, which is deleted in ts+7 (dashed box). Numbers provided refer to the nucleotide position within the HSV-1 genome. (B) Homogeneity of recombinants with rescued ori_L. PCR products were amplified using 2 primer pairs, ICP8-2 and Red-545 (see Fig. 2, line 3), and UL42-2 and UL42-8 as described in the text. The corresponding PCR products are indicated on the right side. Lane 1, KOS/F-B; 2, ts+7/F-C; 3, ts+7/F-Red; 4, rescue-A; 6, rescue-B. The DNA marker is λ DNA digested with *Bst*EII. (C) PCR products of the ori_L-containing DNA fragment, amplified by PCR using primers ICP8-2 and oriL-3′. Lanes 1 to 5: PCR products; lanes 6 to 10: PCR products digested with *Psi*I. Lanes 1 and 6: KOS/F-B; 2 and 7: ts+7/F-A; 3 and 8: ts+7/F-C; 4 and 9: rescue-A; 5 and 10: rescue-B. Lanes 2, 3, 7, and 8 are loaded with one-tenth volume (0.8 μl) of total PCR products relative to others (8 μl). The DNA marker (M) is a 50-bp DNA ladder (New England Biolabs).

Rescue recombinants replicate DNA with wild type level of mutation frequency

A second set of *SupF* mutagenesis experiments (Table 2, Exp. 2) was then performed to measure the mutation frequencies of the *SupF* gene by replicated various recombinants, including the rescue recombinants. Results demonstrated that rescue-A and -B recombinants replicated the *SupF* gene with the frequencies of 0.0123% and 0.0104%, respectively, comparable to KOS/F-B virus (0.0097%), while both ts+7/F-A and -C recombinants exhibited approximately 5-fold lower frequencies [0.0026% ($P < 0.01$) and 0.0020% ($P < 0.05$), respectively] than KOS/F-B virus. Therefore, the increased fidelity mediated by ts+7/F-A and -C recombinants is not due to the effect of second site mutation in the viral genome; the absence of ori_L sequences in these recombinants plays the critical role in replicating DNA with high fidelity. However, it is possible that the inserted 2 Cs upstream of the UL29 in ts+7 recombinants may contribute to the high fidelity of DNA replication.

Recombinant with the ori_L deletion and 11 Cs replicates DNA with a higher fidelity

To examine whether the 13 Cs present in ts+7/F recombinants contribute to the increased DNA replication fidelity of the *SupF* gene, a recombinant virus, ts+7/F-11C, was constructed to contain only 11 Cs and a deletion of the 148-bp ori_L core sequences. Sequencing of the PCR products amplified from virus-infected cell DNA confirmed the correct construct of the recombinant virus. The *SupF* mutagenesis assay was performed and results (Table 2, Exp. 3) demonstrated that the recombinant ts+7/F-11C replicated the *SupF* gene with the mutation frequency of 0.0029%, comparable to ts+7/F-A virus (0.0028%), which contained the 13 Cs, while the KOS/F-B recombinant replicated the *SupF* gene with a significant higher mutation frequency [0.013% ($P < 0.05$)]. Therefore, the insertion of 2 Cs within the run of 11 Cs does not contribute to the observed higher fidelity of the *SupF* genes replicated by the ori_L deletion recombinants. The *SupF* mutagenesis studies clearly demonstrate that recombinant with the intact ori_L

sequences does not replicate DNA as accurate as that without the ori_L sequences and that the ori_L sequences may have an effect in maintaining a certain degree of infidelity during viral DNA replication.

Implications

In this study, the effect of a deletion in the ori_L sequence on replication fidelity was examined. Results clearly demonstrated that HSV-1 recombinants lacking an intact ori_L sequence replicated DNA with a higher fidelity compared to those with the wild type ori_L sequence. It is intriguing that HSV contains three *ori* copies, yet recombinant viruses lacking either ori_L or the two copies of ori_S can replicate efficiently in cultured cells (Igarashi et al., 1993; Polvino-Bodnar et al., 1987). However, mutants lacking both copies of ori_S exhibit a slow accumulation of viral DNA during the early stages of infection and a modest reduction in progeny yields compared to those infected with wild type virus, despite nearly equal amount of total viral DNA synthesized in infected cells (Igarashi et al., 1993). These differences in HSV phenotype require further detailed quantitative investigations to examine the impact of the lack of both copies of ori_S in viral replication efficiency. Studies of ts+7 also demonstrate that the ori_L sequence is not required for virus replication in vitro or in vivo, as this mutant can establish and maintain latent infection, and reactivate from latency (Polvino-Bodnar et al., 1987). However, ts+7 has a decreased burst size relative to its KOS strain (96 vs.136) and forms slightly smaller plaques than KOS virus (Polvino-Bodnar et al., 1987). Although these differences may not be significant, it seems that the presence of all three copies of origins is required for efficient DNA replication. Therefore, careful examinations of the replication efficiencies, such as amounts of DNA synthesized and the growth curves, of ts+7 relative to its parental strain KOS viruses are necessary. The information will be important to imply whether the impact of the ori_L deletion in viral replication efficiency is co-related to the increased fidelity observed in this study.

It has been shown that ori_L and ori_S respond differently to nerve growth factor (NGF) and dexamethasone (DEX) in differentiated or undifferentiated PC12 cells (Hardwicke and Schaffer, 1997), suggesting the different roles for ori_L and ori_S in the HSV life cycle. The results reported in this study also suggest that maintaining ori_L in the HSV genome may have an important role in HSV biology, perhaps to maintain a certain degree of infidelity during DNA replication and viral propagation. It will be interesting to examine whether mutants lacking both copies of ori_S have an effect on DNA replication fidelity.

Another important question is to determine the mechanism responsible for the effect of ori_L on replication fidelity. One possible explanation is that the virus may use two mechanisms to synthesize a fixed amount of total DNA in infected cells, which can be expressed as the amount of DNA derived from the number of origin based initiation of DNA replication times the number of unit length DNA (processive DNA synthesis) from each initiation event. Recombinant virus lacking one origin will result in a decreased number of independent initiation accompanied with an increased number of processive synthesis. Therefore, there are fewer errors resulted from the processive replication. This hypothesis can be tested by measuring the amount of DNA synthesized in infected cells, and by examining whether recombinant lacking both copies of ori_S also replicates DNA with a higher fidelity. Another possibility is that ori_L may affect promoters at long distance, which may lead to altered expressions of other replicative proteins and indirectly affect the replication fidelity. Further studies, including the application of microarray experiments, may provide such information.

Materials and methods

Cells and viruses

Vero and PolA5 cells were grown and maintained as described previously (Hwang et al., 1997). The characteristics of recombinant viruses tkLTRZ1, which contains integrated *Lac Z* gene under the control of Moloney murine leukemia virus LTR promoter (LTR) in the *tk* locus (Davar et al., 1994) (kindly provided by Dr. D.M. Coen), and KOS/F-B, which contains integrated *SupF* amplicon in the *tk* locus (derived from the wild type strain, KOS) (Hwang et al., 2002), have been previously described. Recombinant viruses ts+7/Lac Z-A and -B were constructed from mutant ts+7 (Polvino-Bodnar et al., 1987) with the β-galactosidase (*Lac Z*) gene integrated in the thymidine kinase (*tk*) locus. Recombinant viruses ts+7/F-A and -C were constructed from mutant ts+7 with the *SupF* mutagenesis cassette inserted into the *tk* locus. Recombinant virus ts+7/F-Red2 was constructed from ts+7/F-C with the ori_L and N-terminal portion of the *UL30* gene replaced with the *Pcmv-Red2* sequence, which contains the *DsRed2* gene (isolated from pDsRed2; BD Biosciences) under the control of the CMV IE promoter (Pcmv) (isolated from pCMVβ; BD Biosciences). Rescue-A and -B, and ts+7/F-11C recombinants, were constructed from ts+7/F-Red2 with the *Pcmv-Red2* sequence replaced with the wild type ori_L and the deleted ori_L sequences, respectively, together with the N-terminal portion of *UL30*. These recombinant viruses were propagated in Vero cells, except ts+7/F-Red2, which was propagated in PolA5 cells.

Plasmids

Plasmids ptkLTRZ1 (Davar et al., 1994) (kindly provided by D.M. Coen) and pSupF-tk2 (Hwang and Hwang, 2003) have been described previously. The plasmid series of pGEM-ts+7/Lac Z-UL29 and pGEM-ts+7/F-UL29 were constructed as the following. A 5.0-kb *Avr*II–*Bgl*II DNA fragment, which contains the entire *UL29* open reading frame, ori_L, and the 5′ portion of *UL30*, was isolated from infectious DNA of ts+7/Lac Z-A, -B, and ts+7/F-C recombinants, respectively, and subcloned into the *Xba*I–*Bam*HI site of the pGEM7Zf(+) vector. The following oligonucleotides ICP8-3 (corresponding to nucleotides 60,030 to 60,014 of the HSV-1 genome), ICP8-4 (59,849 to 59,864), ICP8-5 (61,290 to 91,273), ICP8-3688 (61,192 to 61,211), oriL-

3′ (62,680 to 62,661), and T7 primer were used to sequence the entire UL29 sequence.

Plasmid pUL29/30-Red2 (Fig. 2, line 3) was constructed in the following way. A 1.2-kb DNA fragment, containing the N-terminal portion of *UL29* and a partial ori_L sequence (corresponding to nucleotides 61,188 to 62,419 of the HSV-1 genome), was amplified from HSV-1 genomic DNA by PCR using primers ICP8-*Hin*dIII (5′-CCCAAGCTTCGAAGG-CCGTGAACGTAATG-3′) and OriL-*Sph*I (5′-ACATG-CATGCCACGCCCACCGGCTGATGAC-3′). The PCR products were cloned into the pCRT7/NT-TOPO vector (Invitrogen) to construct plasmid pUL29-OriL. A 1.2-kb *Sph*I–*Eco*RI DNA fragment, containing the *Pcmv-Red2* sequence, was cloned into the corresponding restriction sites in pUL29-OriL to construct pUL29-OriL-Red2. A 2.4-kb *Eco*RI fragment was isolated from pGEM3-pol (Hwang et al., 1997), which contains the entire *pol* open reading frame, and was inserted into UL29-OriL-Red2 to construct pUL29/30-Red2. This plasmid contains 1.2 kb of UL29/Ori_L sequence and 2.4 kb of the *pol* gene sequence flanking the *Pcmv-Red2* cassette for subsequent construction of recombinant viruses expressing DsRed2 protein. All restriction enzymes used in this study were from New England Biolabs.

The plasmid pKS-UL29/30 (Fig. 2, line 5) was constructed to contain the 4.4-kb *Cla*I–*Not*I DNA fragment [isolated from viral genomic DNA of the wild type strain KOS (nucleotides 61,396 to 65,771)] and cloned into the pBluescript II KS (+) vector (Stratagene). Recombination-negative *E. coli* Sure 2 host cells (Stratagene) were used to propagate plasmid DNA to prevent the loss of ori_L sequence (Hardwicke and Schaffer, 1995).

The plasmid pKS-UL29/30-ΔOri_L was constructed by the following procedures. First a 514-bp DNA fragment was amplified from ts+7/Lac Z-A-infected cell DNA using both ICP8-2 and oriL-3′ primers. The PCR products were cleaved by *Bsr*GI and *Bam*HI enzymes to obtain a 164-bp DNA fragment, which lacked the ori_L core sequences. This DNA fragment was then used to replace the corresponding 312-bp fragment within pKS-UL29/30 DNA clone. The run of 11 Cs is located upstream of the *Bsr*GI restriction site; therefore, the resulting plasmid pKS-UL29/30-ΔOri_L contains the run of 11 Cs and the deletion of ori_L.

Construction of recombinant viruses

Recombinant viruses tkLTR-Z1 and KOS/F-B (Fig. 1B, line1) were described previously (Davar et al., 1994; Hwang et al., 2002). To construct ts+7/Lac Z recombinant virus, the plasmid ptkLTRZ1, which contains the *Lac Z* gene under control of the Moloney murine leukemia virus LTR promoter (MoMLV-LTR) (Davar et al., 1994), was co-transfected with ts+7 infectious viral DNA into 5×10^5 Vero cells using LipofectAmine 2000, according to the manufacturer's protocol (Invitrogen). Recombinants were isolated by plaque assay using Vero cells, appearing as blue plaques after X-Gal staining. Two independent recombinants, ts+7/Lac Z-A and -B (Fig. 1A), were isolated from independent transfection experiments and twice plaque-purified to homogeneity. Two independent recombinants, ts+7/F-A and -C (Fig. 2, line 2), were constructed by co-transfection of pSupF-tk2 and ts+7 infectious viral DNA into Vero cells. Recombinant viruses were isolated and purified under 10 μM gancyclovir (GCV) selection as described previously (Hwang and Hwang, 2003).

To construct the rescue recombinants, pUL29/30-Red2 was transfected into PolA5 cells using LipofectAmine 2000, followed by superinfection with ts+7/F-C recombinant virus. Progeny viruses were identified, isolated, and purified from plaques expressing red fluorescence using an inverted fluorescent microscope (Nikon Eclipse TE300). Recombinant ts+7/F-Red2 was purified to homogeneity and confirmed by PCR as described below. Plasmid pKS-UL29/30, which contains wild type *UL29*, ori_L, and *UL30* sequences, was transfected into Pol A5 cells, followed by superinfection with ts+7/F-Red recombinant virus. Progeny viruses were identified and isolated by plaque assay as those losing the expression of red fluorescence. Two recombinants, rescue-A and -B, were constructed from two independent transfection experiments, purified to homogeneity, and verified by PCR amplification of the target sequences.

Recombinant virus ts+7/F-11C was constructed by transfection of pKS-UL29/30-Δori_L DNA, which contained the deletion of the ori_L core sequences and 11Cs, into Pol A5 cells using LipofectAmine 2000, followed by superinfection with ts+7/F-Red2 virus. Recombinant viruses were identified and isolated as described above. The lack of ori_L sequences and the presence of 11 Cs was confirmed by sequencing the PCR products amplified from the viral infected cell DNA.

Southern analysis, PCR amplification, and sequencing

Southern analysis was performed as described previously (Hwang et al., 2002). PCR was performed using the following program, except as noted below. PCR amplification was performed in a 50-μl reaction containing 0.5 μg of infected cell DNA, 1 μM of each primer, 200 μM of each dNTP, 5 units *Taq* polymerase (Eppendorf), PCR buffer (Eppendorf), and 10% glycerol. The reaction conditions included a 2-min incubation at 94 °C, 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. For PCR amplification of the ori_L-containing sequences, an annealing temperature of 65 °C was applied, and primers ICP8-2 (5′-TTGATGGTGGTTG-CCGTCTTG-3′) and oriL-3′ (5′-GCAAGGGCCTTGT-TTGTCTG-3′), corresponding to sequences located upstream and downstream of the ori_L sequence (Fig. 2, line 6), were used. PCR was used to amplify the *pol* gene from ts+7/F-A and rescue-A-infected cell DNA using the primer sets described previously (Hwang et al., 2004). The following primers were used to amplify *UL29-DsRed2*: ICP8-2 and Red-545 (5′-AGACAGCTTCTTGTAGTCGGGG-3′, corresponding to nucleotides 533 to 545 of the *DsRed2* coding sequence) (Fig. 2, lines 3 and 6). Primers UL42-2 (5′-GAGGAGGGC-GTGTCGTCCAGCA-3′; corresponding to nucleotides 675 to 696 of the *UL42* open reading frame) and UL42-8 (5′-GTCTTAGGTTTCTTTAGGG-3′; nucleotides 1243 to 1225)

were used to amplify a partial sequence of the *UL42* gene. PCR products were sequenced using an ABI 3100 Genetic Analyzer (Applied Biosystems) at the Upstate Medical University DNA Core Facility or using an ABI PRISM® 3730XL Analyzer at Macrogen. PCR products containing the ori_L sequences were sequenced using the fmol DNA cycle sequencing kit (Promega, Madison), a ^{32}P-labeled oriL-3′ primer, and a modified annealing temperature of 72 °C.

Mutagenesis assays

SupF mutagenesis assay was used to examine the *supF* mutation frequency replicated by the KOS/F, ts+7/F, and rescued recombinant viruses, as described previously (Hwang and Hwang, 2003; Hwang et al., 2002, 2004). Briefly, 100–200 pfu of the recombinant viruses was inoculated into 1×10^5 Vero cells seeded in a culture tube. After absorption for 1 h, infected cells were washed with serum-free DMEM and incubated for 72 h. DNA was then extracted from the infected cells, purified, and digested with *Bam*HI. After self-ligation of *Bam*HI-cleaved DNA, an aliquot of DNA was electroporated into the *E. coli* host strain MBM7070. Transformants were then spread on LB agar plates containing ampicillin, IPTG, and X-Gal. White and light blue colonies were identified as mutants containing the mutated *SupF* gene, and the relative mutation frequency was determined as the ratio of the number of white plus light blue colonies to the total number of colonies.

Recombinant viruses tkLTRZ1, ts+7/Lac Z-A, and -B were used to examine the mutation frequency of the *Lac Z* gene as previously described (Hwang et al., 2003). Briefly, 100 to 200 pfu of recombinant virus was inoculated into 2×10^5 Vero cells. Progeny viruses were harvested at 72 h-post-infection. Approximately 1200 to 1500 pfu of the progeny viruses was plaque assayed on Vero cells seeded on 10-cm culture plate. The cells were overlaid with 0.75% methylcellulose at 1 h-post-infection. At 48 h-post-infection, the cells were fixed and stained with X-gal using the β-galactosidase reporter gene staining kit (Sigma). Clear foci (plaques) were identified by inverted microscopy and mutation frequency was determined as the ratio of the number of clear plaques to the total number of plaques. A chi-square test was used to calculate the statistical significance of the differences in mutation frequencies.

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